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UNITED STATES PATENT APPLICATION

OF

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FOR

DETECTION OF A GENE, *vat*E, ENCODING AN

ACETYLTRANSFERASE INACTIVATING STREPTOGRAMIN

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BACKGROUND OF THE INVENTION

[001] This application claims the right to priority based on Provisional Patent Application No. 60/146,141 filed July 30, 1999. The entire disclosure of Provisional Patent Application No. 60/146,141 is hereby incorporated by reference.

[002] This invention relates to the discovery of a new gene, *vat*E, encoding an acetyltransferase inactivating streptogramin A, which is widely distributed in virginiamycin-resistant *enterococcus faecium* strains.

[003] Streptogramin, virginiamycin, pristinamycin, and synergistin are produced by streptomyces, and consist of synergistic mixtures of two chemically different molecules: A and B compounds (10) In some European countries and in Algeria, these mixtures are used both orally and topically, mostly against staphylococcal infections. Virginiamycin is used as growth promoter in animal feed in Europe and in the U.S.A. Virginiamycin-resistant *Enterococcus faecium* are prevalent in fecal and intestinal samples from turkeys, pigs, broilers, and farmers in Europe and America (1, 14, 19, 20). Since bacteria can be transferred via food from animals to humans, this is alarming, in particular because quinupristin/dalfopristin (J. Antimicrob. Agents Chemother., 1992, 30[suppl.30]), an injectable mixture of semi-synthetic streptogramins soon to be commercialized (Synercid), is expected to be widely used, mainly to treat vancomycin-resistant *E. faecium* infections.

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[004] The *satA* gene (18) encoding an acetyltransferase inactivating A compounds was isolated from an *E. faecium* plasmid. It was found in only 29 % of the 140 tested *E. faecium* strains isolated in Dutch and Danish farms and resistant to

the mixtures (13, 14). Five of the *E. faecium* strains isolated in Denmark harbored a large plasmid conferring resistance to the mixture and which was transferable by filter mating experiments to an *E. faecium* recipient (14). None of the transconjugants harboring these plasmids carried *sat*A, *vat, vat*B, *vga*, or *vga*B (14). These results suggested that the *E. faecium* strains contained other unidentified streptogramin A resistance gene(s). Thus, there continues to exist a need in the art for the identification of new genes specific for *Enterococcus faecium* resistant to streptogramin A and related compounds.

SUMMARY OF THE INVENTION

[005] Accordingly, this invention aids in fulfilling this need in the art by providing a new gene, *vat*E, encoding an acetyltransferase inactivating Streptogramin A, which is widely distributed in virginiamycin-resistant *Enterococcus faecium* strains. In particular, this invention provides a purified peptide-comprising the complete amino acid sequence (SEQ ID NO:1) encoded by the *vat*E gene. This invention also provides polypeptide fragments derived from SEQ ID NO:1 containing at least 10 amino acids. The fragments can be common to all virginiamycin A acetyl transferases as shown on SEQ ID NOS:5, 6, 7, and 8. The fragments according to the invention can be specific of as shown on SEQ ID NOS:9, 10, 11, and 12.

[006] This invention additionally provides a purified polynucleotide comprising the complete nucleic acid sequence of the *vat*E gene (SEQ ID NO:2). This invention also provides nucleic acid fragments derived from SEQ ID NOS:3 and 4 containing 15 to 40 nucleotides as primers F and R. For example, the fragments

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are those corresponding to nucleotides No. 899 to 878 and to nucleotides No. 354 to nucleotide 378. (Fig. 1)

[007] In addition, two primers have been selected in order to obtain, after using an amplification technique and after cloning the amplified sequence, the complete gene of *vat*E capable of being expressed in *Staphylococcus aureus* (strain No. 4220 described by KREISWIRTH et al. in Nature 1983, Vol. 306, pp. 709-712]. These primers are shown in SEQ ID NOS:13 and 14 as *vat*EA and *vat*EB. The *vat*EA is from nucleotide 98 to nucleotide 120 (Figure 1). The *vat*EB is from nucleotide 982 to nucleotide 957 (Figure 1).

[008] In SEQ ID NOS:13 and 14, an artificial site was created if comparing with the original sequence in Figure 1 at nucleotides No. 107 and 109 where G was replaced by T and G was replaced by C, respectively. An *Eco*RI site was then produced in the first primer. In the same manner, in SEQ ID NOS:3 and 4, at nucleotide No. 362, A replaces G (as in the original sequence) and at nucleotide 367, C replaces G (as in the original sequence). A new *Eco*RI site was created. In the second primer of SEQ ID NOS:3 and 4, a new *Eco*RI site was created by replacing at nucleotide No. 891 G by C and on the complementary strand (as shown) the base C (original) is replaced by G.

[009] This invention also provides a composition comprising purified polynucleotide sequences including at least one nucleotide sequence of the genes selected from the group consisting of synthetic polynucleotides or fragments of genes or cDNA of *vat*E useful for the detection of resistance to streptogramin A and related compounds. The gene *vat*E was obtained from a *Hind*III fragment of 5 kb

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prepared from *enterococcus faecium* genome (strain K14) after digestion by *Hind*III restriction enzyme. The *Hind* III fragment hybridizes with an amplicon containing two degenerated or consensus primers referred to as M and N, which are defined as SEQ ID NOS:5, 6, 7, and 8. This amplicon has 147 nucleotides. The amplicon or the two degenerated primers (M and N) can be used for the preparation of DNA chips as taught in PCT applications No. W095.11.995 and No. WO 97.02.357. The sequences upstream and downstream of this amplicon were obtained.

[010] The DNA fragment containing the *vat*E gene including the amplicon is shown in Figure 1. A region having in this said fragment, the possible properties of a bacterial promoter activity is located as follows in Figure 1. Its sequence is:

```
                                                    -35
                                                  TGTCACA
                                                    -10
201   ACTACTTATT TTTTTACCCA ATCTTCTAGA  CTATAAT
                                 XbaI
```

(SEQ ID NO:16)

[011] Additionally, the invention includes a purified polynucleotide that hybridizes specifically under stringent conditions with a polynucleotide of SEQ ID NOS: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12.

[012] The invention further includes polynucleotide fragments comprising at least 10 nucleotides capable of hybridization under stringent conditions with any one of the nucleotide sequences enumerated above.

[013] In another embodiment of the invention, a recombinant DNA sequence comprising at least one nucleotide sequence enumerated above and under the control of regulatory elements that regulate the expression of resistance to

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antibiotics of the streptogramin family in a defined host is provided. The amplified complete gene of *vat*E, including the amplicon and the promoter, is shown in SEQ ID NO:15.

[014] Furthermore, the invention includes a recombinant vector comprising the recombinant DNA sequence noted above, wherein the vector comprises the plasmid pIP 1801 contained in *E. coli*. The recombinant strain has been deposited at the collection C.N.C.M. in Paris, France, under the accession number I-2247 on July 7, 1999.

[015] The invention also includes a recombinant cell host comprising a polynucleotide sequence enumerated above or the recombinant vector defined above.

[016] In still a further embodiment of the invention, a method detecting bacterial strains that contain the polynucleotide sequences set forth above is provided.

[017] Additionally, the invention includes kits for the detection of the presence of bacterial strains that contain the polynucleotide sequences set forth above.

[018] The invention also contemplates antibodies recognizing peptide fragments or polypeptides encoded by the polynucleotide sequences enumerated above.

[019] Still further, the invention provides for a screening method for active antibiotics and/or molecules for the treatment of infections due to Gram-positive bacteria, particularly enterococci, based on the detection of activity of these

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antibiotics and/or molecules on bacteria having the resistance phenotype to streptogramins.

BRIEF DESCRIPTION OF THE DRAWINGS

[020] This invention will be more fully described with reference to the drawings in which:

[021] FIGURE 1 discloses the complete nucleotide and amino acid sequences of *vat*E. In this Figure are also represented the upstream and downstream regions of the *vat*E gene.

[022] FIGURE 2 is a comparison between the sequences of *vat*E protein and four acetyltransferase enzymes already published.

[023] FIGURE 3 is a restriction map of the insert of 1080 bp contained in the plasmid deposited in *E. coli* at C.N.C.M. I-2247.

DETAILED DESCRIPTION OF THE INVENTION

[024] The present invention pertains to polynucleotides derived from *Enterococcus faecium* genes encoding resistance to streptogramin A and chemically related compounds. This invention also relates to the use of the polynucleotides as oligonucleotide primers or probes for detecting *Enterococcus faecium* strains that are resistant to streptogramin A and related compounds in a biological sample.

[025] In another embodiment, the present invention is directed to the full length coding sequences of the *Enterococcus faecium* genes encoding resistance to streptogramin A and to the polypeptides expressed by these full length coding sequences.

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[026] Further, this invention relates to the use of the expressed polypeptides to produce specific monoclonal or polyclonal antibodies that serve as detection means in order to character any *Enterococcus faecium* strain carrying genes encoding resistance to streptogramin A and chemically related compounds

[027] The present invention is also directed to diagnostic method for detecting specific strains of *Enterococcus faecium* expected to be contained in a biological sample. The diagnostic methods use the oligonucleotide probes and primers as well as the antibodies of the invention.

[028] More particularly, it has now been determined that bacteria from the *Enterococcus faecium* genus carry a *vat*E gene, which confers resistance to streptogramin A. A gene encoding an acetyltransferase inactivating streptogramin A was isolated from an *Enterococcus faecium* strain and sequenced. The gene, designated *vat*E, encodes a 23,775 kDa protein exhibiting 48.5 to 59.9 % amino acid identity with four other enzymes with the same activity, *vat, vat*B, *vat*C, and *sat*A. The calculation of the percentage of identity was made by using the program gap of GCG software (version 9.1). The parameters are chosen as follows:

a) for amino acid comparisons:

gap penalty: 12

gap extension penalty: 4

length: the sequence to be compared in SEQ ID NO:1 having 213 amino acids.

b) for nucleotide comparisons:

gap penalty: 50

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gap extension penalty: 3.

[029] Figure 2 shows the comparative amino acid alignments of *vat*E with four virginiamycin *A* acetyl transferase proteins. The *sat*B protein is from *Enterococcus,* and *vat*, *vat*B, and *vat*C are from *Staphylococcus.*

[030] Novel polynucleotides corresponding to the *vat*E gene from various strains of *Enterococcus faecium* have been isolated and sequenced. These polynucleotides include SEQ ID NO:2. By "polynucleotides" according to the invention is meant the sequence referred to as SEQ ID NO:2, and the complementary sequences and/or the sequences of polynucleotides that hybridize to the referred sequences in high stringent conditions (hybridization in a mixture containing 5 x SSPE, 5x Denhart solution, 0.5% SDS (w/v) and 100 µg/ml salmon sperm DNA]. The membrane on which is hybridized the DNA, is washed 2 times during 10 minutes, in 2x SSPE, 0.1% SDS (w/v) at room temperature and then the membrane (or the filter) is immersed in a solution of 1 x SSPE, 0.1% SDS (w/v) during 15 minutes at 68°C and finally in a solution of 1 x SSPE, 0.1% SDS (w/v) during 15 minutes at 68°C. The polynucleotides according to the invention are used for detecting *Enterococcus faecium* strains carrying a gene encoding resistance to streptogramin A.

[031] By "active molecule" according to the invention is meant a molecule capable of inhibiting the activity of the purified polypeptide as defined in the present invention or capable of inhibiting the bacterial culture of *Enterococcus faecium* strains.

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[032] Thus, the polynucleotides of SEQ ID NO:2 and its fragment can be used to select nucleotide primers notably for an amplification reaction, such as the amplification reactions further described. PCR is described in the U.S. Patent No. 4,683,202 granted to Cetus Corp. The amplified fragments may identified by agarose or polyacrylamide gel electrophoresis, or by a capillary electrophoresis, or alternatively by a chromatography technique (gel filtration, hydrophobic chromatography, or ion exchange chromatography). The specificity of the amplification can be ensured by a molecular hybridization using as nucleic probes the polynucleotides derived from SEQ ID NO:2 and its fragments, oligonucleotides that are complementary to these polynucleotides or fragments thereof, or their amplification products themselves.

[033] Amplified nucleotide fragments are useful as probes in hybridization reactions in order to detect the presence of one polynucleotide according to the present invention or in order detect the presence of a bacteria of *Enterococcus faecium* strain carrying genes encoding resistance to streptogramin A, in a biological sample. This invention also provides, the amplified nucleic acid fragments ("amplicons") defined herein above. These probes and amplicons can be radioactively or non-radioactively labeled, using for example enzymes or fluorescent compounds.

[034] Preferred nucleic acid fragments that can serve as primers according to the present invention are the following in the Fig. 1:

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PRIMER F 5' -CAATATTGGAATTCGGGACTACACC-3' (SEQ ID NO:3)
*Eco*RI
nt 354 nt 378 gene *vat*E

PRIMER R 5' -CTGTTTATGAATTCAAGTGTGG-3' (SEQ ID NO:4)
*Eco*RI
nt 899 nt 878 gene *vat*E

[035] The primers can also be used as oligonucleotide probes to specifically detect a polynucleotide according to the invention.

[036] Other techniques related to nucleic acid amplification can also be used and are generally preferred to the PCR technique. The Strand Displacement Amplification (SDA) technique (Walker et al., 1992) is an isothermal amplification technique based on the ability of a restriction enzyme to cleave one of the strands at a recognition site (which is under a hemiphosphorothioate form), and on the property of a DNA polymerase to initiate the synthesis of a new strand from the 3' OH end generated by the restriction enzyme and on the property of this DNA polymerase to displace the previously synthesized strand being localized downstream.

[037] The SDA amplification technique is more easily performed than PCR (a single thermostated water bath device is necessary and is faster than the other amplification methods. Thus, the present invention also comprises using the nucleic acid fragment according to the invention (primers) in a method of DNA or RNA amplification according to the SDA technique. The polynucleotides of SEQ ID NO:2 and its fragments, especially the primers according to the invention, are useful as technical means for performing different target nucleic acid amplification methods such as:

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[038] - TAS (Transcription-based Amplification System), described by Kwoh et al. in 1989;

[039] - SR (Self-Sustained Sequence Replication), described by Guatelli et al. in 1990;

[040] - NASBA (Nucleic acid Sequence Based Amplification), described by Kievitis et al. in 1991; and

[041] - TMA (Transcription Mediated Amplification).

[042] The polynucleotides of SEQ ID NO:2 and its fragments, especially the primers according to the invention, are also useful as technical means for performing methods for amplification or modification of a nucleic acid used as a probe, such as:

[043] - LCR (Ligase Chain Reaction), described by Landegren et al. in 1988 and improved by Barany et al. in 1991, who employ a thermostable ligase;

[044] - RCR (Repair Chain Reaction), described by Segev et al. 1992;

[045] - CPR (Cycling Probe Reaction), described by Duck et al. in 1990; and

[046] - Q-beta replicase reaction, described by Miele et al. in 1983 and improved by Chu et al. in 1986, Lizardi et al. in 1988 and by Burg et al. and Stone et al. in 1996.

[047] When the target polynucleotide to be detected is RNA, for example mRNA, a reverse transcriptase enzyme can be used before the amplification reaction in order to obtain a cDNA from the RNA contained in the biological sample. The generated cDNA can be subsequently used as the nucleic acid target for the

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primers or the probes used in an amplification process or a detection process according to the present invention.

[048] Nucleic probes according to the present invention are specific to detect a polynucleotide of the invention. By "specific probes" according to the invention is meant any oligonucleotide that hybridizes with the polynucleotide of SEQ ID NO:2, and which does not hybridize with unrelated sequences. Preferred oligonucleotide probes according to the invention are SEQ ID NOS:5, 6, 7, or 8 or SEQ ID NOS:3 or 4.

[049] In a specific embodiment, the purified polynucleotides according to the present invention encompass polynucleotides having at least 80% identity in their nucleic acid sequences with polynucleotide of SEQ ID NO:2. By percentage of nucleotide homology according to the present invention is intended a percentage of identity between the corresponding bases of two homologous polynucleotides, this percentage of identity being purely statistical and the differences between two homologous polynucleotides being located at random and on the whole length of said polynucleotides. The calculation was made according to the software GCG and the program "gap."

[050] The oligonucleotide probes according to the present invention hybridize specifically with a DNA or RNA molecule comprising all or part of the polynucleotide of SEQ ID NO:2 under stringent conditions. As an illustrative embodiment, the stringent hybridization conditions used in order to specifically detect a polynucleotide according to the present invention are advantageously the following:

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[051] Prehybridization and hybridization are performed at 68°C a mixture containing:

- 5X SSPE (1X SSPE is .3 M NaCl, 30 mM tri-sodium citrate
- 5X Denhardt's solution
- 0.5% (w/v) sodium dodecyl sulfate (SDS); and
- 100 µg ml^{-1} salmon sperm DNA

[052] The washings are performed as follows:

- Two washings at laboratory temperature for 10 min. in the presence of 2 x SSPE and 0.1 % SDS;
- One washing at 68°C for 15 min. in the presence of 1 x SSPE, .1% SDS; and
- One washing at 68°C for 15 min. in the presence of 0.1 x SSPE and 0.1 % SDS.

[053] The non-labeled polynucleotides or oligonucleotides of the invention can be directly used as probes. Nevertheless, the polynucleotides or oligonucleotides are generally labeled with radioactive element (^{32}P, ^{35}S, ^{3}H, ^{125}I) or by a non-isotopic molecule (for example, biotin, acetylaminofluorene, digoxigenin, 5-bromodesoxyuridin, fluorescein) in order to generate probes that are useful for numerous applications. Examples of non-radioactive labeling of nucleic acid fragments are described in the French Patent No. FR 78 10975 or by Urdea et al. or Sanchez Pescador et al. 1988.

[054] Other labeling techniques can also be used, such as those described in the French patents 2 422 956 and 2 518 755. The hybridization step may be

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performed in different ways (Matthews et al. 1988). A general method comprises immobilizing the nucleic acid that has been extracted from the biological sample on a substrate (nitrocellulose, nylon, polystyrene) and then incubating, in defined conditions, the target nucleic acid with the probe. Subsequent to the hybridization step, the excess amount of the specific probe is discarded, and the hybrid molecules formed are detected by an appropriate method (radioactivity, fluorescence, or enzyme activity measurement).

[055] Advantageously, the probes according to the present invention can have structural characteristics such that they allow signal amplification, such structural characteristics being, for example, branched DNA probes as those described by Urdea et al. in 1991 or in the European Patent No. 0 225 807 (Chiron).

[056] In another advantageous embodiment of the present invention, the probes described herein can be used as "capture probes", and are for this purpose immobilized on a substrate in order to capture the target nucleic acid contained in a biological sample. The captured target nucleic acid is subsequently detected with a second probe, which recognizes a sequence of the target nucleic acid that is different from the sequence recognized by the capture probe.

[057] The oligonucleotide fragments useful as probes or primers according to the present invention can be prepared by cleavage of the polynucleotide of SEQ ID NO:2 by restriction enzymes, as described in Sambrook et al. in 1989. Another appropriate preparation process of the nucleic acids of the invention containing at most 200 nucleotides (or 200 bp if these molecules are double-stranded) comprises the following steps:

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- synthesizing DNA using the automated method of beta-cyanethylphosphoramidite described in 1986;
- cloning the thus obtained nucleic acids in an appropriate vector; and
- purifying the nucleic acid by hybridizing to an appropriate probe according to the present invention.

[058] A chemical method for producing the nucleic acids according to the invention, which have a length of more than 200 nucleotides (or 200 bp if these molecules are double-stranded) comprises the following steps:

- assembling the chemically synthesized oligonucleotides having different restriction sites at each end;
- cloning the thus obtained nucleic acids in an appropriate vector; and
- purifying the nucleic acid by hybridizing to an appropriate probe according to the present invention.

[059] The oligonucleotide probes according to the present invention can also be used in a detection device comprising a matrix library of probes immobilized on a substrate, the sequence of each probe of a given length being localized in a shift of or several bases, one from the other, each probe of the matrix library thus being complementary to a distinct sequence of the target nucleic acid. Optionally, the substrate of the matrix can be a material able to act as an electron donor, the detection of the matrix positions in which hybridization has occurred being subsequently determined by an electronic device. Such matrix libraries of probes and methods of specific detection of a target nucleic acid are described in the European patent application No. 713 016, or PCT Application No. WO 95 33846, or

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also PCT Application No. WO 95 11995 (Affymax Technologies), PCT Application No. WO 97 02357 (Affymetrix Inc.), and also in U.S. Patent No. 5,202,231 (Drmanac), said patents and patent applications being herein incorporated by reference.

[060] The present invention also pertains to a family of recombinant plasmids containing at least a nucleic acid according to the invention. According to an advantageous embodiment, a recombinant plasmid comprises a polynucleotide of SEQ ID NO:2 or nucleic acid fragment thereof. More specifically, the following plasmid is part of the invention: pIP1801 or its fragments. Said fragments are derived from the use of restriction enzymes according to the restriction map of the gene *vat*E, as shown in Figure 3.

[061] The present invention is also directed to the full length coding sequences of the *vat*E gene from *Enterococcus faecium* available using the purified polynucleotides according to the present invention, as well as to the polypeptide enzymes encoded by these full length coding sequences. In a specific embodiment of the present invention, the full length coding sequence of the *vat*E gene is isolated from a plasmid or cosmid library of the genome of *Enterococcus faecium* that has been screened with the oligonucleotide probe according to the present invention. The selected positive plasmid or cosmid clones hybridizing with the oligonucleotide probes of the invention are then sequenced in order to characterize the corresponding full length coding sequence, and the DNA insert of interest is then cloned in an expression vector conferring resistance to streptogramin A and related compounds.

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[062] A suitable vector for the expression in bacteria and in particular in *E. coli*, is the pQE-30 vector (QIAexpress) that allows the production of a recombinant protein containing a 6xHis affinity tag. The 6xHis tag is placed at the C-terminus of the recombinant polypeptide ATP binding motif conferring resistance to streptogramin A and related compounds.

[063] The polypeptides according to the invention can also be prepared by conventional methods of chemical synthesis, either in a homogenous solution or in solid phase. As an illustrative embodiment of such chemical polypeptide synthesis techniques the homogenous solution technique described by Houbenweyl in 1974 may be cited.

[064] The polypeptide conferring resistance to streptogramin A and related compounds is useful for the preparation of polyclonal or monoclonal antibodies that recognize the polypeptides or fragments thereof. The monoclonal antibodies can be prepared from hybridomas according to the technique described by Kohler and Milstein in 1975. The polyclonal antibodies can be prepared by immunization of a mammal, especially a mouse or a rabbit, with a polypeptide according to the invention that is combined with an adjuvant, and then by purifying specific antibodies contained in the serum of the immunized animal on a affinity chromatography column on which has previously been immobilized the polypeptide that has been used as the antigen.

[065] Consequently, the invention is also directed to a method detecting specifically the presence of a polypeptide according to the invention in a biological sample. The method comprises:

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a) bringing into contact the biological sample with an antibody according to the invention; and

b) detecting antigen-antibody complex formed.

[066] Also part of the invention is a diagnostic kit for *in vitro* detecting the presence of a polypeptide according to the present invention in a biological sample. The kit comprises:

- a polyclonal or monoclonal antibody as described above, optionally labeled; and
- a reagent allowing the detection of the antigen-antibody complexes formed, wherein the reagent carries optionally a label, or being able to be recognized itself by a labeled reagent, more particularly in the case when the above-mentioned monoclonal or polyclonal antibody is not labeled by itself.

[067] Indeed, the monoclonal or polyclonal antibodies according to the present invention are useful as detection means in order to identify or characterize a *Staphylococcal* strain carrying gene encoding resistance to streptogramin A.

[068] The invention also pertains to:

[069] - A purified polypeptide or a peptide fragment having at least 10 amino acids, which is recognized by antibodies directed against a polynucleotide sequence conferring resistance to streptogramin and related compounds, corresponding to a polynucleotide sequence according to the invention.

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[070] - A polynucleotide comprising the full length coding sequence of a *Enterococcus faecium* streptogramin A resistant gene containing a polynucleotide sequence according to the invention.

[071] - A monoclonal or polyclonal antibody directed against a polypeptide or a peptide fragment encoded by the polynucleotide sequences according to the invention.

[072] - A method of detecting the presence of bacterium harboring the polynucleotide sequences according to the invention in a biological sample comprising:

a) contacting bacterial DNA of the biological sample with a primer or a probe according to the invention, which hybridizes with a nucleotide sequence encoding resistance to streptogramins;

b) amplifying the nucleotide sequence using said primer or said probe; and

c) detecting the hybridized complex formed between said primer or probe with the DNA.

[073] A kit for detecting the presence of bacterium having resistance to streptogramin A and harboring the polynucleotide sequences according to the invention in a biological sample, said kit comprising:

a) a polynucleotide probe according to the invention; and

b) reagents necessary to perform a nucleic acid hybridization reaction.

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[074] A kit for detecting the presence of bacterium having resistance to streptogramin A and harboring the polynucleotide sequences according to the invention in a biological sample, said kit comprising:

a) a polynucleotide probe according to the invention; and

b) reagents necessary to perform a nucleic acid hybridization reaction.

[075] A method of screening active antibiotics for the treatment of the infections due to Gram-positive bacteria, comprising the steps of:

a) bringing into contact a Gram-positive bacteria having a resistance to streptogramin A and related compounds and containing the polynucleotide sequences according to the invention with the antibiotic; and

b) measuring an activity of the antibiotic on the bacteria having a resistance to streptogramins and related compounds.

[076] A method of screening for active synthetic molecules capable of penetrating into a bacteria of the family of enterococcus, wherein the inhibiting activity of these molecules is tested on at least a polypeptide encoded by the polynucleotide sequences according to the invention comprising the steps of:

a) contacting a sample of said active molecules with the bacteria;

b) testing the capacity of the active molecules to penetrate into the bacteria and the capacity of inhibiting a bacterial culture at various concentration of the molecules; and

c) choosing the active molecule that provides an inhibitory effect of at least 80% on the bacterial culture compared to an untreated culture.

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[077] An *in vitro* method of screening for active molecules capable of inhibiting a polypeptide encoded by the polynucleotide sequences according to the invention, wherein the inhibiting activity of these molecules is tested on at least said polypeptide, said method comprising the steps of:

a) extracting a purified polypeptide according to the invention;

b) contacting the active molecules with said purified polypeptide;

c) testing the capacity of the active molecules, at various concentrations, to inhibit the activity of the purified polypeptide; and

d) choosing the active molecule that provides an inhibitory effect of at least 80 % on the activity of the said purified polypeptide.

[078] In the inactivation by the O-acetylation of virginiamycin A mediated by *vat*E, the donor of acetyl group is probably acetyl coenzyme A. The acetylation reduced coenzyme A can react with 5,5'-dithio-bis 2 nitrobenzoate (DTNB) to yield 5 thio 2 nitrobenzoate with a 1:1 stoichiometry. The 5 thio 2 nitrobenzoate is yellow and has a high extinction coefficient (E = 00136 mM^{-1} cm^{-1}) at λ 412 nm. Therefore, the measurement of its appearance can be recorded by a spectrophotometer with great sensitivity.

[079] As examples there are disclosed hereinafter the detection of chloramphenicol acetyl transferase activity. Similar conditions may be used for the detection of virginiamycin A activity to yield 5-thio-2-nitrobenzoate with a 1:1 stoichiometry. The thio-2-nitrobenzoate is yellow and has a high extinction coefficient (00136 mM^{-1} cm^{-1}) at λ412 nm. Therefore, the measurement of its appearance can be recorded spectrophotometrically with great sensitivity.

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[080] Materials

- Bacterial strain(s) to be tested.
- Mueller-Hinton (MH) broth (Gram-negative bacteria) or brain heart infusion (BHI) broth (Gram-positive cocci)
- 50 ml centrifuge tubes
- Eppendorf tubes
- Low speed centrifuge
- Refrigerated bench centrifuge
- Sonicator
- Double beam recording spectrophotometer equipped with a water-jacketed cuvette
- Tris-Cl 1M (pH 6.0)
- Tris-Cl 1M (pH 7.8)
- Dithiothreitol (DTT) 0.01 M
- TDTT buffer: Tris-Cl50 mM (pH 7.8)
  DTT 20 µM
- Acetyl coenzyme A (CoASAc) 10 mM (PH 6.0) : dissolve 8.2 mg of CoASAc in 1 ml of 5 mM Tris-Cl (pH 6.0). This solution can be stored frozen for 1 year in aliquots (100 µl).
- 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) 10 mM (PH 7.8) : dissolve 20 µg of DTNB in 0.5 ml of 1 M Tris-C1 (pH 7.8) and add 4.5 ml of distilled water. This solution should be make fresh each time.
- Reaction mixture: CoASAc (10 mM, pH 6.0):100µl

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DTNB (10 mM, pH 7.8):500 µl

Tris-Cl (1 M, pH 7.8):920 µl

H_2O up to 10 ml

This volume is sufficient for 15 reactions.

- Chloramphenicol (Cm) 10 mM (pH 7.8): dissolve 3.2 mg of Cm in 1 ml of 100 mM Tris-Cl (pH 7.8). Heat 10 min in water bath at 100°C. Store at +4°C.

[081] **Protocol**

[082] **Day 1**

[083] 1. Inoculate separately 5 ml of MH broth with bacterial strains to be tested. Grow overnight at 37°C with moderate shaking.

[084] **Day 2**

[085] 2. Inoculate separately 35 ml of MH broth with 1 ml of each overnight culture. Grow at 37°C with moderate shaking until the OD_{600nm} is 0.8.

[086] 3. Centrifuge the cells (8000 rpm, 10 min, 4°C). Discard the supernatant and wash the pellet in 1 ml of TDDT buffer. Centrifuge again and resuspend the pellet in 1 ml of the same buffer.

[087] 4. Sonicate the cells at 4°C with 5 x 30 s pulses with 30 s rest in between.

[088] 5. Centrifuge the samples in an Eppendorf centrifuge (15000 rpm, 10 min, 4°C) to remove cell debris.

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[089] 6. Transfer supernatants (S20) to other Eppendorf tubes and keep on ice until assayed. If the CAT assay is not performed on the same day, freeze the samples at -20°C. The CAT activity can be retained frozen for at least one month.

[090] 7. Pour 600 µl of reaction mixture to reference cuvette and sample cuvettes equilibrated at 37°C in a double beam recording spectrophotometer. Let stand 2 min and adjust OD_{412nm} to 0.

[091] 8. Add 20 µl of S20 to sample cuvettes, mix well, and record OD_{412nm} for 1-2 min to determine the background CAT activity.

[092] 9. When a constant slope is obtained, add 10 µl of Cm to cuvettes, mix well and record the increase in absorbance (OD_{412nm}) for about 5 min. If the CAT activity is too high, a more reliable measure of activity can be obtained by diluting the S20.

[093] 10. Measure the amount of protein in the S20.

[094] 11. Determine the slope (OD_{412nm}/min) before and after adding Cm and then subtract the background slope from the sample slope. The CAT enzyme specific activity expressed in nmole/min/mg is $\Delta\ OD_{412nm}$/0.0136/mg protein [0.0136 being the extinction coefficient ($mM^{-1}cm^{-1}$ λ412nm) of 5-thio-2-nitrobenzoatel.

[095] ***Notes***

[096] - Since the formation of 5-thio-2-nitrobenzoate is accompanied by the appearance of a yellow coloration, CAT activity can be qualitatively appreciated *de visu* in step 9.

[097] - The crude extracts (S20) obtained from certain bacterial genera contain high thioesterase activity that may mask that of CAT since it alsc catalyzes

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the formation of reduced coenzyme A. On the other hand, DTNB was reported to inhibit certain CTAs from Gram-negative bacteria. In both cases, partial purification of the enzyme or the use of an alternate procedure is necessary to overcome these problems. Different methods for convenient CAT assays based on labeled acetyl coenzyme A (or butyryl coenzyme A) for acyl donor are available.

[098] - Chloramphenicol analogs, such as 3'-desoxychloramphenicol, can be used to induce CAT expression in Gram-positive bacteria. This compound is not acetylated by CATs (free inducer) and has little effect on protein synthesis.

[099] - Medium containing carbohydrates other than glucose may be used for the growth of Gram-negative bacteria to avoid catabolic repression.

[0100] If an active molecule for inhibiting the activity of the bacterial enzyme (acetyl transferase) is added to a culture medium containing the resistant strain, the acetyl co-enzyme A present in said medium is not degradated. If the molecule to be tested is not active on the resistant bacteria, the amount of acetyl co-enzyme A decreases.

[0101] A test for screening the inhibiting activity of a molecule, for example, a new antibiotic or a new antibacterial agent, can comprise the following steps:

[0102] a) adding purified active acetyl transferase *vat*E in a solution containing virginiamycin A at various concentrations, acetyl co-enzyme A,

[0103] b) adding the molecule to be tested at various concentrations,

[0104] c) revealing the presence of acetyl co-enzyme A activity and quantifying said acetyl coenzyme A, if necessary, and

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[0105] d) comparing the quantification of acetyl coenzyme A with a control without the new molecule.

[0106] A composition of a polynucleotide sequence encoding resistance to streptogramins and related compounds, or inducing resistance in Gram-positive bacteria, wherein said composition comprises a nucleotide sequence corresponding to the resistance phenotype of the plasmid pIP1807 deposited with the C.N.C.M. under the Accession No. I-2247 on July 7, 1999.

[0107] A method of detecting the presence of bacterium harboring the polynucleotide sequences according to the invention in a biological sample, said method comprising the steps of:

[0108] a) contacting said sample with an antibody

[0109] according to the invention that recognizes a polypeptide encoded by said polynucleotide sequences; and

[0110] b) detecting said complex.

[0111] A diagnostic kit for *in vitro* detecting the presence of bacterium harboring the polynucleotide sequences according to invention in a biological sample, said kit comprising:

[0112] a) a predetermined quantity of monoclonal or polyclonal antibodies according to the invention;

[0113] b) reagents necessary to perform an immunological reaction between the antibodies and a polypeptide encoded by said polynucleotide sequences; and

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[0114] c) reagents necessary for detecting said complex between the antibodies and the polypeptide encoded by said polynucleotide sequences.

[0115] Plasmids containing the polynucleotides from *Enterococcus faecium*, which confer streptogramin A resistance have been inserted into vectors, which have been deposited at the Collection Nationale de Cultures de Microorganismes ("C.N.C.M.") Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris Cedex 15 France on July 7, 1999, as follows:

[0116] Plasmid Accession No.

[0117] pIP1801 I-2247

[0118] This invention will now be described in greater detail in the following Example.

Example

[0119] A collection of 51 *E. faecium* strains was studied for resistance to streptogramins. The strains were isolated from fecal samples from poultry (n = 22), pigs (n = 5), farmers (n = 19) and (sub)urban residents (n = 5) in the Netherlands (Table 1). *sat*A was previously found in 19 strains and *vgb* in a single strain by PCR (14). The *E. faecium* strains were analyzed for nucleotide sequences hybridizing at high stringency (65°C) with the eight genes previously found in staphylococcal and enterococcal plasmids conferring resistance to the mixtures: *satA* (18), *vat* (9), *vat*B (3), *vat*C (6), *vga* (7) and *vga*B (4) conferring resistance to A compounds and the two genes, *vgb* (8) and *vgbB*(6), encoding lactonases hydrolyzing B compounds. Nineteen of the strains carried *satA* and the combination of *vat* and *vgb* was

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detected in a single strain, KH6 (Table 1). These two latter genes are contiguous and in the same relative position as in the staphylococcal plasmids in which *vat-vgb* are carried by a DNA fragment originating from the *E. faecalis* plasmid, pAMβ1 (5).

[0120] *Table 1. Relevant characteristics of the 51 E. faecium strains isolated in the Netherlands.*

Strain		*Sg^R genes*	
designation	Origin (city)	Designation (1)	Size (in kb) of the hybridizing *Hind*III fragment (2)
4	pig (Weert)	*vat*E	1.8
14	pig (Weert)	*vat*E	1.8
17	pig (Weert)	satA	4.5*
18	pig (Weert)	satA	3.8*
19	pig (Weert)	satA	3.8*
K12	turkey	*vat*E	6.0*
K13	turkey	*vat*E	5.6*
K14	turkey	*vat*E	6.0*
K15	turkey	*vat*E	5.6*
K36	turkey	satA	6.0*
K40	turkey	*vat*E	3.6*
KS30	turkey	*vat*E	1.4
KS31	turkey	*vat*E	1.4
KS33	turkey	*vat*E	1.4
SK1	broiler	*vat*E	3.2*
SK2	broiler	*vat*E	3.2*
SK3	broiler	*vat*E	> 10*

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Strain		*Sg^R genes*	
designation	Origin (city)	Designation (1)	Size (in kb) of the hybridizing *Hind*III fragment (2)
SK6	broiler	satA	7.0*
SK7	broiler	*vat*E	5.6*
SK8	broiler	*vat*E	5.6*
SK13	broiler	*vat*E	3.2*
SK19	broiler	*vat*E	3.9*
PS17	broiler	*vat*E	4.3*
PS22	broiler	satA	4.0*
PS26	broiler	*vat*E	4.3*
PS35	broiler	*vat*E	4.3*
PS42	broiler	*vat*E	4.3*
KH2	turkey farmer	*vat*E	5.6*
KH4	turkey farmer	satA	3.9*
KH5	turkey farmer	*vat*E	7.3*
KH6	turkey farmer	*vat*E *vat*-vgb	5.6* 8.9
KH7	turkey farmer	*vat*E	2.5
KH15	turkey farmer	satA	2.3*
KH18	turkey farmer	*vat*E	2.3
KH19	turkey farmer	*vat*E	2.3
KH29	turkey farmer	*vat*E	2.3*
KH36	turkey farmer	satA	3.9
KH39	turkey farmer	*vat*E	2.9
LKH2	chicken farmer	satA	4.0*
LKH4	chicken farmer	satA	4.3*

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Strain		Sg^R genes	
designation	Origin (city)	Designation (1)	Size (in kb) of the hybridizing *Hind*III fragment (2)
SKH4	chicken farmer	*vat*E	2.5*
SKH8	chicken farmer	satA	5.6*
SKH11	chicken farmer	*vat*E	2.5
SKH16	chicken farmer	satA	4.5*
SKH18	chicken farmer	satA	3.9*
SKH23	chicken farmer	satA	3.9*
M2	Suburban (Weert)	satA	3.9*
M5	suburban (Weert)	*vat*E	1.9
R2	suburban (Roermond)	satA	3.9*
R24	suburban (Roermond)	satA	3.9*
W3	suburban (Weert)	satA	4.0*

[0121] (1) The strains were screened for Sg^R genes by hybridization at high annealing temperature (65°C) (2) with probe consisting of (i) recombinant plasmids containing DNA inserts from within each of the following genes: *vat*(9), *vat*B (3), *vat*C (6), *vga* (7) or *vga*B (4) or (ii) DNA fragments amplified from *satA* (18) or *vat*E (this study) by PCR with the following pairs of primers: *sat1* (nt position: 189-210 in *satA*, Acc. No. L12033) and *sat*2 (nt position: 760-782 in *sat*_ or *vat*E-F (nt position: 354-378 in *vat*E, Acc. No. AF153312) and *vat*E-R (nt position: 878-899 in *vat*E).

[0122] (2) The *Hind*III fragments indicated with an asterisk were detected in extrachromosomal DNA bands (≥ 40 kb) migrating above the chromosomal DNA

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fragments of the uncleaved total cellular DNA, in agarose gel electrophoresis in Tr-acetate buffer. In the other strains, the hybridizing bands comigrated with the chromosomal fragments, but the hybridization signals were as strong as those of the extrachromosomal DNA, suggesting that they may be carried plasmids.

[0123] Thirty-one of the tested *E. faecium* strains did not contain any of the eight genes investigated. PCR experiments were carried out at low annealing temperature (40°C) with a pair of degenerate primers, M and N (3, 16), designed to amplify a DNA fragment from any sequence encoding a streptogramin A acetyltransferase containing two well conserved motifs, III and IV (3, 6, 16). A DNA fragment of the expected size (147 nt) was amplified from the cellular DNA of all the strains. The amplicon obtained with the strain K14 was sequenced using oligonucleotides M and N as primers. Its sequence was only 60.4 % to 68.6% similar to the SgA acetyltransferase genes (*vat*, *vat*B, *vat*C, *satA*), suggesting that the amplicon was from a different gene. A 5 kb *Hind*III fragment hybridizing with the sequenced amplicon was isolated from the cellular DNA of strain K14 and inserted into the *Hind*III site of pUC18. The resulting plasmid, pIP1798, was used to sequence 1080 nt of the insert including the sequences hybridizing with the 147 bp amplicon.

[0124] The sequence (registered in the GenBank EMBL data Library under Accession No. AF153312) contains a 642 bp gene including an ATG start codon preceded, 6 nt upstream, by a putative ribosome-binding site. The free energy of association of the most stable structure between this site and the 3' terminus of the 16S rRNA was -61.5 kj/mol. This gene, named *vat*E, is similar to those encoding

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SgA acetyltranferases, *satA*, *vat*, *vat*B, and *vat*C (54.3, 58.0, 60.0, and 60.1 % similarity, respectively). *vat*E encodes a putative 214 aa protein of 23,775 Da similar to xenobiotic acetyltranferases (17). It is most similar to the SgA acetyltranferases, *Sat*A, *Vat*, *Vat*B, and *Vat*C (48.5, 50.0, 59.9 and 50.9 % identical amino acids, respectively).

[0125] Most *vat*-related genes in staphylococcal plasmids are contiguous to and downstream from another streptogramin-resistance (Sg^R) gene. The pairs of genes are probably co-transcribed (12). However, analysis of the 270 and 170 nt sequences flanking *vat*E did not suggest the presence of any contiguous Sg^R gene.

[0126] A DNA fragment of 858 nt containing *vat*E (nt 104 to nt 961, Accession No. AF153312) was amplified from pIP1798 and inserted between the *Eco*RI and *Sma*I sites of the shuttle vector, pOX7 (11). The resulting plasmid, pIP1801, introduced by electroporation in the *S. aureus* recipient, RN4220 (15), conferred resistance to pristinamycin IIA (MICs: 2 µg/ml for RN4220 [pOX7] and 8 µg/ml for RN4220 [pIP1801]).

[0127] The presence of *vat*E in other strains was tested by hybridization experiments at high stringency. Nucleotide sequences hybridizing with *vat*E-probe were detected in the 32 strains which did not carry *satA*, including the strain containing *vat-vgb* (Table 1). Total cellular DNA of strain KH6 was subjected to agarose gel electrophoresis. The *vat*E and *vat-vgb* sequences migrated to different positions, suggesting that they are not carried by the same plasmid.

[0128] The distribution of the streptogramin-resistance genes in the collection of *E. faecium* studied was clearly different from that found in staphylococci

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(2). It is worth checking whether the high prevalence of *vat*E in this collection, is also observed among infectious clinical isolates.

[0129] In summary, the present invention pertains to polynucleotides derived from *Enterococcus faecium* genes encoding resistance to streptogramin A and chemically related compounds. This invention also relates to the use of the polynucleotides as oligonucleotide primers or probes for detecting *Enterococcus faecium* strains that are resistant to streptogramin A and related compounds in a biological sample.

[0130] In another embodiment, the present invention is directed to the full length coding sequences of the *Enterococcus faecium* genes encoding for resistance to streptogramin A and to the polypeptides expressed by these full length coding sequences.

[0131] Further, this invention relates to the use of the expressed polypeptides to produce specific monoclonal or polyclonal antibodies that serve as detection means in order to characterize any *Enterococcus faecium* strain carrying genes encoding resistance to streptogramin A and chemically related compounds.

[0132] The present invention is also directed to diagnostic methods for detecting specific strains of *Enterococcus faecium* expected to be contained in a biological sample. The diagnostic methods use the oligonucleotide probes and primers as well as the antibodies of the invention raised against *Vat*E protein or its fragments.

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[0133] The invention relates also to a method of screening of molecules, which are capable to inactivate the acetyl transferase activity in bacteria. A bacterial culture, which is resistant to virginiamycin A, can grow in the presence of virginiamycin, but cannot grow if a new molecule active against acetyl transferase activity is added to the culture medium.

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